AD_____
(Leave blank)

Award Number: DAMD17-99-1-9413

TITLE: The Role of EMMPRIN in Tumor Progression

PRINCIPAL INVESTIGATOR: Bryan P. Toole, Ph.D.

CONTRACTING ORGANIZATION: Tufts University
Boston, Massachusetts, 02111

REPORT DATE: May, 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. Agency Use Only (Leave blank)	2. Report Date	3. Report Type and Period Covered (i.e., annual 1 Jun 00 - 31 May 01)
	May, 2001	Annual; 1 May, 00 - 30 April, 01

4. Title and Subtitle
The Role of EMMPRIN in Tumor Progression

5. Award Number
DAMD-17-99-1-9413

6. Author(s)
Bryan P. Toole, Ph.D.

7. Performing Organization Name (Include Name, City, State, Zip Code and Email for Principal Investigator)
Tufts University
Boston, Massachusetts, 02111

E-Mail: bryan.toole@tufts.edu

8. Performing Organization Report Number (Leave Blank)

9. Sponsoring/Monitoring Agency Name and Address
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. Sponsoring/Monitoring Agency Report Number (Leave Blank)

11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)

12a. Distribution/Availability Statement (check one)
Approved for public release; distribution unlimited

12b. Distribution Code
(Leave Blank)

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Crucial steps in tumor progression and the process of metastasis, e.g. tumor growth, invasion through extracellular matrice and angiogenesis, involve proteolytic modification of the pericellular matrix surrounding tumor cells. A major class o proteases involved in these processes is the matrix metalloproteinases MMPs), and inhibition of MMPs prevent progression and metastasis of several tumor types, including human breast carcinomas, in animal models. In vivo, tumo MMPs are usually produced by stromal cells associated with tumors rather than the tumor cells themselves. The tumor cel surface glycoprotein, EMMPRIN, stimulates MMP production by fibroblasts and endothelial cells, and may be an importan regulator of MMP production during tumorigenesis in vivo. However no direct evidence for an important role in tumo progression has been published. The focus of this proposal will be to demonstrate directly whether or not EMMPRI promotes breast cancer progression, whether a role for EMMPRIN in tumor progression may be to promote or induc angiogenesis, and whether inhibitory peptides can be developed that may have future therapeutic potential. This study shoul determine definitively whether EMMPRIN-mediated regulation of MMPs may constitute a newly discovered step in breas carcinoma progression and metastasis. Interference with EMMPRIN action may then be an effective way to retard breas carcinoma progression in patients

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Breast cancer; metastasis; angiogenesis; peptide antagonists

15. Number of Pages (count all pages including appendices) 36

16. Price Code

17. Security Classification of Report	18. Security Classification of this Page	19. Security Classification of Abstract	20. Limitation of Abstract
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Crucial steps in tumor progression and the process of metastasis, e.g. tumor growth, invasion through extracellular matrices and angiogenesis, involve proteolytic modification of the pericellular matrix surrounding tumor cells. A major class of proteases involved in these processes is the matrix metalloproteinases (MMPs), and inhibition of MMPs prevents progression and metastasis of several tumor types, including human breast carcinomas, in animal models. In vivo, tumor MMPs are usually produced by stromal cells associated with tumors rather than the tumor cells themselves. The tumor cell surface glycoprotein, EMMPRIN, stimulates MMP production by fibroblasts and endothelial cells, and may be an important regulator of MMP production during tumorigenesis in vivo. However no direct evidence for an important role in tumor progression has been published. The focus of this proposal will be to demonstrate directly whether or not EMMPRIN promotes breast cancer progression and whether a role for EMMPRIN in tumor progression may be to promote or induce angiogenesis. This study should determine definitively whether EMMPRIN-mediated regulation of MMPs may constitute a newly discovered step in breast carcinoma progression and metastasis. Interference with EMMPRIN action may then be an effective way to retard breast carcinoma progression in patients.

BODY

Original Statement of Work Task 1: To document that increased expression of EMMPRIN (extracellular matrix metalloproteinase inducer) in non-aggressive human breast carcinoma cells leads to increased tumor growth, blood vessel formation and metastasis.

As proposed in our original Statement of Work, we have tested whether tumor growth and metastasis are affected when EMMPRIN expression is increased in non-aggressive MDA-MB436 human breast carcinoma cells. In previous studies we demonstrated that EMMPRIN stimulates production of matrix metalloproteinase (MMP) production in fibroblasts, possibly explaining the finding that most MMPs are produced by tumor stromal fibroblasts rather than by tumor cells themselves. We have now shown that EMMPRIN promotes tumor progression in vivo. Human MDA-MB-436 breast cancer cells, which are slow growing in vivo, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female nu/nu mice. Green fluorescent protein (GFP) was used to visualize metastases. Breast cancer cell clones transfected with EMMPRIN/GFP cDNA gave rise to much larger tumors than GFP- or plasmid-transfected cancer cells, although the EMMPRIN transfectants grew at the same rate as controls under routine culture conditions. The EMMPRIN transfectants were more invasive both in vivo and in in vitro invasion assays, and gave rise to metastases in approximately 25% of the experimental animals. Control animals showed no signs of invasiveness or metastases. Mortality was 100% in experimental and insignificant in controls over a 15-week period. Increased MMP expression was also demonstrated in EMMPRIN-enhanced tumors. Details of these experiments are given in a manuscript in press and attached in Appendix 2.

Further extension of this work, as outlined in the original Statement of Work will performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work has been approved as of March 23, 2000 (see Appendix 1).

Original Statement of Work Task 2: To document whether inhibition of EMMPRIN expression in malignant human and murine mammary carcinoma cells blocks tumor growth and/or metastasis in vivo.

As proposed, antisense cDNA and ribozyme constructs were produced for mouse and human EMMPRIN, as well as sense controls. Stable transfectants of murine TA3/St mammary carcinoma cells (an aggressive cancer cell line) were produced using these constructs. Unfortunately, complete inhibition of EMMPRIN expression has not yet been obtained. Clones were isolated that exhibited partially inhibited expression and these were used to determine whether metastasis to the lung after intravenous injection into the tail of syngeneic mice was affected. These experiments have not been effective. Continuation of this work will performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work has been approved as of March 23, 2000 (see Appendix 1).

Original Statement of Work Task 3: To test whether EMMPRIN stimulates endothelial morphogenesis in a 3-dimensional collagenous matrix in culture.

We have set up the methods and have performed initial experiments that support our proposal. In these experiments, human umbilical vein endothelial cells were cultured on type I collagen gels, then treated with either bFGF, a known angiogenic agent, or purified EMMPRIN. This method is a standard technique that mimics aspects of angiogenesis, i.e. invasion of a three-dimensional matrix and formation of capillary-like tubules. In our experiments we have observed that 1 μg of EMMPRIN duplicates the effect of 5 ng of bFGF in its ability to initiate capillary-like tubule formation. We believe that the relatively high amount of EMMPRIN required is due to inactivation of most of the protein during purification. For this reason and since EMMPRIN has now been shown to bind collagenase (MMP-1) and to be present in some EMMPRIN preparations (see Appendix 3), we have switched to a new technique for studying this phenomenon.

We have found that infection of fibroblasts with recombinant EMMPRIN adenovirus is a very effective means to stimulate MMP production, due to mutual interaction between neighboring cells expressing both EMMPRIN and EMMPRIN receptor. We are now using this approach with human endothelial cells. That is, we infect endothelial cells with the adenovirus, then monitor expression of EMMPRIN and MMPs. We will determine the effect of the adenoviral-delivered EMMPRIN on capillary tubule formation. This approach will now be supported by new funds from the National Cancer Institute (CA79866). See newly approved Statement of Work (Appendix 1).

Revised Statement of Work (see Appendix 1) Task 1: To test the effects of a peptide antagonist of EMMPRIN action on tumor growth and invasion in animal tumor models.

We have attempted to map the active site of the EMMPRIN molecule by testing the effect on MMP production of synthetic peptides with sequences from within the extracellular domain of the EMMPRIN molecule. The aim of this approach was to determine whether synthetic peptides from the active site would antagonize or mimic the action of EMMPRIN. Previous results suggested that we had obtained an inhibitory peptide. However it turns out that the activity of the

peptide depends on the way in which it is tested; it is inhibitory in cocultures of tumor cells and fibroblasts but stimulatory when applied directly to fibroblasts alone. We are currently attempting to understand this unexpected finding.

Despite the confusing results above, the active peptides have been useful for ligand chromatographic isolation of an EMMPRIN-binding protein. This protein has been identified as annexin II. We are currently attempting to determine the role of EMMPRIN-annexin II interaction in EMMPRIN action.

Revised Statement of Work Task 2: To explore the use of recombinant adenoviral constructs for efficient delivery.

We have begun to produce recombinant adenoviruses driving expression of EMMPRIN and mutated forms of EMMPRIN to further map the active site of EMMPRIN. In addition these adenoviruses can be used for delivery in tests of EMMPRIN function. In this way we have found that an adenoviral construct driving expression of a soluble form of EMMPRIN (lacking transmembrane and cytoplasmic domains) lacks EMMPRIN activity and may act as a dominant negative inhibitor. We will futher test its ability to block EMMPRIN function.

KEY RESEARCH ACCOMPLISHMENTS

1) Demonstration that increased expression of EMMPRIN leads to increased tumor growth and invasion in vivo
2) Development of an efficient recombinant adenovirus-based system for testing the cellular effects of EMMPRIN and EMMPRIN mutants
3) Tentative identification of the "active site" for EMMPRIN action
4) Demonstration of specific binding of EMMPRIN to collagenase (MMP-1)
5) Demonstration of specific binding of EMMPRIN to annexin II

REPORTABLE OUTCOMES

1) Publication:

Guo H, Li R, Zucker S, Toole BP: EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. Cancer Res. **60**, 888-891 (2000).

2) Manuscript in press:

Zucker S, Hymowitz M, Rollo EE, Mann R, Connor CE, Cao J, Foda HD, Tompkins DC, Toole BP: Tumorigenic potential of extracellular matrix metalloproteinase inducer (EMMPRIN). Amer. J. Pathol, in press.

3) Presentations:

FASEB conference symposium on Misregulation of the Basement Membrane in Human Disease, April 2000: "Regulation of tumor progression by EMMPRIN, a tumor cell surface inducer

of matrix metalloproteinases" by Bryan Toole

Plenary Lecture to the Finnish Cancer Society, October, 2000: "Emmprin and hyaluronan-tumor cell interactions: new targets for therapeutic intervention?" by Bryan Toole

Symposium on Membrane Proteases and Cancer, Sicily, May, 2001: "Emmprin, a tumor cell surface inducer of MMP production, promotes tumor progression" by Bryan Toole

4) New funding;

National Cancer Institute (CA 79866): "Tumor cell-stromal interactions in cancer"

CONCLUSIONS

We conclude from the above work that EMMPRIN promotes mammary carcinoma progression via stimulation of MMP production in tumor stromal cells. Our preliminary data suggest that a mechanism whereby EMMPRIN may act is via stimulation of angiogenesis and also, possibly, by presentation of interstitial collagenase on the tumor cell surface. Our recent data suggest that EMMPRIN-derived peptides or soluble forms of EMMPRIN may be efficacious as inhibitors of EMMPRIN action and therefore may be useful therapeutically.

REFERENCES: None

APPENDIX 1

Revised Statement of Work (DAMD17-99-9413) - approved March 23, 2000

Title: Role of EMMPRIN in Tumor Progression
P.I.: Bryan P. Toole

In work supported by DAMD17-99-9413, a peptide with amino acid sequence identical to part of the outer immunoglobulin domain of EMMPRIN has been shown to inhibit stimulation of matrix metalloproteinase production by EMMPRIN in cell culture. Also, we have shown that relatively benign MDA-MB436 human mammary carcinoma cells express low levels of EMMPRIN, and that stable transfection of MDA-MBA436 cells with cDNA for EMMPRIN leads to expression of high levels of EMMPRIN; these transfectants grow much more aggressively in vivo than control MBA436 cells, have become invasive, and give rise to high mortality rates (S.Zucker, M.Hymowitz, E.Rollo, R.Mann, C.Conner, J.Cao, H.Foda, D.Tompkins, B.Toole: in press; **Appendix 2 of this report**).

Task 1: To test the effects of a peptide antagonist of EMMPRIN action for its potential inhibitory effect on tumor growth and invasion in animal tumor models.

- set up two animal tumor models to test effect of peptide antagonist: a) MDA-MB**231** metastatic human mammary carcinoma cells; b) EMMPRIN transfectants of benign MDA-MB**436** cells (see above). The tumor cells will be implanted in mammary fat pads of nude mice and monitored for tumor growth and invasion, as described in original application.
- use mini-osmotic pumps to deliver peptide antagonist or control peptide (scrambled antagonist sequence) to area of mammary carcinoma growth, using methods previously employed (C.Zeng, B.Toole, S.Kinney, J.Kuo, I.Stamenkovic, Int. J. Cancer 77:396-401, 1998).

Task 2: To explore use of recombinant adenoviral constructs for efficient delivery of the peptide antagonist and to determine effects on tumor growth and metastasis. We will explore this avenue in case delivery by mini-osmotic pump as proposed above does not provide sufficient concentration of peptide to be maximally effective.

- design and construct recombinant adenoviruses containing cDNA for peptide antagonist fused to carrier protein prepro-region (to ensure appropriate processing and secretion as active peptide), using methods already developed for construction of wild type and mutated EMMPRIN adenoviruses in work supported by DAMD17-99-9413.
- test efficacy of recombinant adenoviral constructs in driving expression and secretion of active peptide in cultured target cells.
- test recombinant adenovirus in tumor models above, using ex vivo infection in culture prior to implantation or in situ injection of virus into tumor in vivo.
- explore the effect of co-injection of recombinant virus on formation of metastatic nodules in the lung on intravenous injection of tumor cells.

APPENDIX 2 (American Journal of Pathology, In press)

Tumorigenic Potential of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)[1]

Stanley Zucker[2], Michelle Hymowitz, Ellen E. Rollo, Richard Mann, Cathleen E. Connor, Jian Cao, Hussein D. Foda, David C. Tompkins, and Bryan P. Toole[3]

Departments of Research and Medicine, Veterans Affairs Medical Center, Northport, NY 11768; State University of New York at Stony Brook, NY 11794; and [3]Tufts University School of Medicine, Boston, MA 02111

Running Title: EMMPRIN is tumorigenic

Key Words: matrix metalloproteinases (MMPs); cancer invasion; metastasis

[1]Supported by Department of Defense Breast Cancer Research Program Grants (DAMD 17-95-5017 and DAMD 17-99-9413) and a Department of Veteran Affairs Merit Review Grant

[2]To whom requests for reprints should be addressed

Abbreviations: ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer; GFP, green fluorescent protein; MMP, matrix metalloproteinase

ABSTRACT

Extracellular Matrix MetalloPRoteinase INducer (EMMPRIN), a glycoprotein present on the cancer cell plasma membrane, enhances fibroblast synthesis of matrix metalloproteinases (MMPs). The demonstration that peritumoral fibroblasts synthesize most of the MMPs in human tumors rather than the cancer cells themselves, has ignited interest in the role of EMMPRIN in tumor dissemination. In this report we have demonstrated a role for EMMPRIN in cancer progression. Human MDA-MB-436 breast cancer cells, which are tumorigenic but slow growing in vivo, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female NCr nu/nu mice. Green fluorescent protein (GFP) was utilized to visualize metastases. In three experiments, breast cancer cell clones transfected with EMMPRIN cDNA were considerably more tumorigenic and invasive than plasmid-transfected cancer cells. Increased gelatinase A and gelatinase B expression (demonstrated by in situ hybridization and gelatin substrate zymography) was demonstrated in EMMPRIN-enhanced tumors. In contrast to de novo breast cancers in humans, human tumors transplanted into mice elicited minimal stromal or inflammatory cell reactions. Based on these experimental studies and our previous demonstration that EMMPRIN is prominently displayed in human cancer tissue, we propose that EMMPRIN plays an important role in cancer progression by increasing synthesis of MMPs.

INTRODUCTION

Extracellular Matrix MetalloPRoteinase INducer (EMMPRIN) was originally designated Tumor Collagenase Stimulating Factor (TCSF) by Biswas et al. following isolation and purification of the 58 kDa glycoprotein from the plasma membrane of cancer cells and demonstration of its function in stimulating fibroblast synthesis of collagenase-1 (MMP-1).[1] The subsequent finding that EMMPRIN also induced fibroblast synthesis of gelatinase A (MMP-2) and stromelysin-1 (MMP-3) indicated a more general effect on production of MMPs.[2] Recent studies have documented the capacity of recombinant EMMPRIN or EMMPRIN purified from cancer cells to stimulate fibroblast production/secretion of stromelysin-1, collagenase-1, and gelatinase A in vitro.[2, 3] Following secretion from fibroblasts, collagenase-1 is able to bind to EMMPRIN on the tumor cell surface.[4] The demonstration by in situ hybridization (mRNA localization) that peritumoral fibroblasts synthesize most of the MMPs (collagenases, gelatinases, stromelysins, MT-MMPs) in human tumors rather than the cancer cells themselves has ignited interest in the role of EMMPRIN in tumor dissemination.[5, 6] The association of intense EMMPRIN expression in neoplastic cells within invasive human tumors[7,8] further supports a role for EMMPRIN in cancer dissemination. These data are consistent with a central function for EMMPRIN in stimulating stromal cell production of MMPs which, following pericellular activation, directly degrade the extracellular matrix (ECM).[1]

Peptide sequencing and cDNA isolation of EMMPRIN from tumor cells [1,9] led to the recognition that EMMPRIN is identical to human basigin [10] and M6 antigen,[11] proteins of previously unknown function that were identified by other investigators in embryonic and inflammatory tissues. A knockout mouse has been produced in which the murine homologue of basigin/EMMPRIN is lacking.[12] The null mutant is in most cases unable to undergo oocyte implantation, presumably due to the requirement for MMPs in this process. It is apparent that although many embryonic and adult tissues express EMMPRIN, the level of EMMPRIN expression and glycosylation in tumors is much greater than in corresponding normal tissues.

In the current study we have examined the function of EMMPRIN in a cancer model in immunodeficient mice. Human MDA-MB-436 breast cancer cells which are tumorigenic, estrogen independent, moderately invasive in vitro, but slow growing in vivo,[13] were transfected with EMMPRIN cDNA and injected orthotopically into the mammary fat pad of nude mice. We took advantage of the observation that the 29 kDa green fluorescent protein (GFP) of the jelly fish Aequoria victora retains its fluorescent properties when recombinantly expressed in eukaryotic cells[14] along with EMMPRIN cDNA and can be used as a powerful marker for gene expression and cancer dissemination in vivo. Cancer cells transfected with both EMMPRIN cDNA and GFP cDNA were compared to cancer cells transfected with GFP cDNA alone for tumorigenic behavior. The results demonstrated that tumor growth in nude mice was considerably enhanced by EMMPRIN/GFP transfected breast cancer cells as compared to GFP-alone transfected cells.

MATERIALS AND METHODS

Reagents

Restriction enzymes were purchased from Stratagene (La Jolla, CA). EMMPRIN was purified from LX-1 lung cancer cells using affinity column chromatography.[15] Monoclonal antibodies to EMMPRIN (clone 1G6.2) were produced in collaboration with Dr. Dembro at Chemicon, International, Inc. (Temecula, CA). The F4/80 rat anti-mouse macrophage antibody was purchased from Serotec (Raleigh, NC). Thrombin was a kind gift from Dr. J. Jesty. Phorbol 12-myristate-13 acetate (PMA) was purchased from Sigma (St. Louis, MO).

Cell lines and Culture Conditions

Human MDA-MB-436 breast cancer cells were maintained in Richter's Improved Minimal Essential Medium supplemented with 10% donor calf serum.[13] Immunostaining of MDA-MB-

436 cells was performed using a primary mouse monoclonal antibody to EMMPRIN (1G6.2) and a secondary goat anti-mouse IgG (H&L) horseradish peroxidase labeled antibody.

Construction of Plasmids and Transfection into Cells

A 1.6 kb cDNA[1], representing the entire EMMPRIN sequence encoding 269 amino acid residues, was placed at an EcoR I site under the control of the CMV promoter in pcDNA3 (Invitrogen, Carlsbad, CA). To facilitate identification of transfected cells in vitro and metastases in vivo, green fluorescent protein (GFPmut1 variant) cDNA (Clontec Lab, Inc., Palo Alto, CA) was inserted into the EMMPRIN-containing plasmid. The GFP cDNA along with a separate upstream CMV promoter from pEGFP-C1 plasmid (Clontec Lab, Palo Alto, CA) was inserted into the EMMPRIN expression vector between Not I and Xho I sites as shown in Figure 1A. An additional polyadenylation (PA) signal from pSG5 (Stratagene) was placed downstream of the EMMPRIN gene to provide balanced expression of both recombinant genes under control of CMV promoters. The resulting plasmid was named EMMPRIN/GFP. As a control plasmid, GFP cDNA alone was subcloned into pcDNA3 without EMMPRIN cDNA. In experiment 2, EMMPRIN cDNA was subcloned into pcDNA3 without GFP; the control plasmid was pcDNA3 alone.

The human MDA-MB-436 breast cancer cell line was stably transfected using the calcium phosphate precipitation method.[16] Selected G418-resistant clones were screened by fluorescent appearance using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set. Fluorescent positive clones were further analyzed by Northern blot analysis probed with an EMMPRIN cDNA fragment.

RNA Isolation and Northern Blot Hybridization

Total RNA was extracted from MDA-MB-436 cells stably transfected with desired plasmids by guanidine solubilization, phenol/chloroform extraction, and serial precipitation. [1,17] Approximately 20 μg of total RNA was resolved by denaturing gel electrophoresis followed by

Northern transfer to nylon membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized to ^{32}P-radiolabeled EMMPRIN cDNA (1.7 Kb) at 42°C as described [17] and analyzed after overnight exposure with an intensity screen at -80°C. The amount of the samples applied to the lanes was normalized by β-actin RNA.

Labeling of RNA Probes

Antisense and sense digoxigenin (DIG)-labeled RNA probes for EMMPRIN, gelatinase A, and gelatinase B were synthesized by reverse transcribing 1μg of cDNA from a PCR reaction that had used gene-specific primers that contain the T7 or T3 phage promoter sequence followed by 20-25 bases of the mRNA sequence.[18] The probes for human EMMPRIN (bases 319-701), human gelatinase A (bases 42-436), and mouse gelatinase B (bases 56-361) were designed based on published nucleotide sequences (GenBank Accession # AH007299, #J03210, and #Z27231, respectively). Homology between the human and mouse nucleotide sequences for gelatinase A and gelatinase B are 91% and 78%, respectively, as determined by BLAST 2 sequence alignment (www.ncbi.nlm.nih.gov/gorf/bl2.html). *In vitro* transcription of the amplified DNA template was performed using the digoxigenin RNA labeling kit (Roche Molecular Biochemicals). Labeled probes were purified and sequences were verified.

In situ hybridization

Serial sections of paraffin-embedded mouse tumors were prepared for *in situ* hybridization according to the method of Komminoth.[19] Slides were processed for immunodetection employing anti-DIG alkaline phosphatase antibody and then incubated with substrate solution (Wash and Block Set, Roche Molecular Biochemicals).

Cell Proliferation In Vitro

Cell proliferation assays were performed by plating MDA-MB-436 cells at 4 X 10^4 cells per well (Costar, Corning, NY) and then switched to serum-free media. After 48 hours, serum

enriched media was added back and cells were cultivated for 4 additional days. Cell counts were performed daily.

Tumor Formation in Mice and Preparation of Tissue Extracts

Four week old female athymic NCr nu/nu mice were obtained from Taconic Farms (Germantown, NY). Cancer cells (1×10^6) were injected into the mammary fat pad of nude mice. Tumor growth was monitored weekly. Tumor volume was calculated using the formula: $(\text{length})(\text{width})^2/2$. At termination of experiments, mice were sacrificed, autopsied, and tissue sections of the primary tumor, lungs, liver, lymph nodes, gastrointestinal tract, and other suspicious areas were prepared for histologic/microscopic examination (hematoxylin-eosin staining of paraffin-embedded sections) . Tissue sections were also stored in liquid nitrogen for subsequent in situ hybridization (see above) and extraction of MMPs. The extraction procedure for tumor tissue involved detergent and heat-extraction steps.[20]

Immunohistochemistry, Gelatin Substrate Zymography, and Protein Studies

Primary cell cultures were transferred to serum-free media and cultivated for 18 hours with or without the addition of thrombin or PMA. Spent media was then collected and tested by gelatin zymography. Gelatin substrate zymography was preformed in 10% polyacrylamide gels that had been cast in the presence of 0.1% gelatin (NOVEX, San Diego, CA).[21, 22] Protein determinations were made using the bicinchoninic acid reagent (Pierce, Rockford, IL).

Immunohistochemistry for mouse macrophages, monocytes, and dendritic cells was performed using the F4/80 rat antibody as described by Tsuruga, et al. [23] A biotinylated rabbit anti-rat IgG was used as the secondary antibody. Immunoreactivity was visualized by the avidin-biotin peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

Analysis of variance and Student's t-test were employed to compare differences between groups in various experiments; $p<0.05$ was considered significant. Survival experiences between groups were compared by the Wilcox chi-square test.

RESULTS

Cell Transfection and Proliferation

Northern blot analysis using EMMPRIN cDNA as a probe detected 20 fold enhanced EMMPRIN expression by EMMPRIN/GFP-transfected cells as compared to GFP or non-transfected cells (Figure 1B). Immunostaining of MDA-MB-436 cells using specific mouse monoclonal antibodies to EMMPRIN documented intense staining of EMMPRIN/GFP transfected cells and infrequent weak staining of GFP-transfected or vector transfected cells (data not shown).

There were no significant differences in cell doubling times between GFP and EMMPRIN/GFP cDNA transfected cells (~18 hours) in media with or without serum. This data is inconsistent with EMMPRIN acting as an autocrine growth factor for tumor cells in vitro.

Tumor Growth in Nude Mice

Three independent experiments, each employing a different clone of EMMPRIN-transfected MDA-MB-436 cells, were performed. In experiments 1 and 2, the GFP-alone or vector transfected clones did not form palpable tumors by the time of the experiment's termination at 12 weeks; however, ~.01 cm^3 non-invasive tumors were identified at autopsy in 18/18 mice. In contrast, the EMMPRIN/GFP or EMMPRIN (alone) transfected clones formed palpable breast tumors at the site of mammary injection by week 6 in 18/18 mice which grew progressively to >1.7 cm^3 in diameter by week 12 at which time the animals were sacrificed. Histologic examination of tissue sections revealed local cancer invasion, but no metastases. EMMPRIN/GFP and GFP expressing tumors expressed green fluorescence when examined grossly with a fluorescent light.

Experiment 3: Groups of 10 mice were injected with transfected MDA-MB-436 cells into mammary tissue. The tumors emanating from the EMMPRIN/GFP cDNA transfected MDA-MB-436 cells grew relatively rapidly and all mice expired or had to be sacrificed within 12 weeks (Figure 2A); extensive metastases to the liver, lung, pleura, spleen, lymph nodes, and

mesentery were present in 3/10 mice. In contrast, injection of the GFP cDNA transfected tumor cells into mice resulted in tumors that grew considerably more slowly than EMMPRIN/GFP expressing tumors. Tumor diameter was <0.3 cm^3 and no metastases were noted at week 15 in 9 of 10 GFP transfectant mice. One mouse in the GFP-transfected group developed a 1.4 cm^3 primary tumor by week 12.

Gelatinolytic Activity Extracted from Tumor Tissues and Cells

Gelatin zymograms of conditioned media from 18 hour cultivated MDA-MB-436 tumor cells (Figure 2B- left panel) revealed that cells transfected with EMMPRIN/GFP cDNA secreted >3 fold more progelatinase A (72 kDa) than GFP cDNA transfected cells. Treatment of both sets of transfected cells with thrombin (20 nM) enhanced both secretion and activation of progelatinase A (more prominently displayed in the EMMPRIN/GFP transfected cells). Treatment of cells with PMA (100 nM) resulted in the appearance of weak gelatinolytic bands at 92 kDa consistent with human progelatinase B.

Extracts of tumors derived from EMMPRIN/GFP cancer cell injections in mice displayed intense gelatinolytic bands localized at 105 kDa, 92 kDa, 85 kDa, 72 kDa, and 64-62 kDa (Figure 2B- right panel). The 105 kDa band is consistent with mouse latent gelatinase B; human latent gelatinase B and activated mouse gelatinase B migrates at ~92 kDa.[24] The 72 kDa and 62 kDa gelatinolytic bands could represent human or mouse latent and activated gelatinase A, respectively. Tumor extracts from GFP alone-injected mice revealed weaker gelatinolytic bands (with minimal activated gelatinolytic bands) than EMMPRIN/GFP injected mice.

Histochemistry/In situ Hybridization

Hematoxylin and eosin staining of resected breast masses revealed extensive replacement of normal mammary tissue with carcinoma in tumors originating from mice injected with EMMPRIN/GFP or GFP cDNA-transfected MDA-MB-436 cells; other than size of the tumor masses, the EMMPRIN-transfected and vector-transfected tumors were indistinguishable by

routine staining. Minimal fibrosis and inflammatory cell infiltration were noted in tumor tissue and surrounding normal-appearing mammary tissue. The sparsity of inflammatory cells in the tumors was confirmed using an antibody (F4/80) which recognizes mouse macrophages, monocytes, and dendritic cells (data not shown).

In situ hybridization of tumor tissue from six mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining for EMMPRIN mRNA in cancer cells (Figure 3, panel 2). Surrounding normal-appearing mammary ductal cells and scattered periductal cells also expressed EMMPRIN mRNA (panel 6). Gelatinase A mRNA was found in both cancer cells (panel 3) and the surrounding non malignant tissue including normal-appearing mammary ducts and adipose cells (panel 7). There was specific staining for gelatinase B mRNA in the tumor sections (panel 4), but not as widely distributed as gelatinase A. By counting the number of stained cells in serial sections of EMMPRIN cDNA-transfected tumors, the ratio of cells immunotyped as macrophages (F4/80 antibody) versus gelatinase B mRNA expressing cells was ~1:70. Intense staining for gelatinase B was also noted in small aggregates of cells (negative staining for mouse macrophages using F4/80 antibody) scattered around normal-appearing ducts (panel 8). Similar in situ hybridization results were found on examination of metastatic tumors in the EMMPRIN/GFP treated mice (data not shown). Similar results were achieved employing either human or mouse gelatinase A and gelatinase B mRNA probes. Specific staining was abolished by pretreatment of tissues with RNAase (data not shown). No staining was detected in any of the tumor tissues that were hybridized with EMMPRIN, gelatinase A, or gelatinase B sense probes (data not shown).

In the GFP alone-transfected tumors (seven mice examined), virtually no EMMPRIN, gelatinase A, or gelatinase B mRNA was identified in the tumor cells or in the surrounding normal appearing mammary tissue (Figure 3- panels 10-12 and 14-16).

DISCUSSION

The current report describes a direct effect of EMMPRIN expression on tumorigenicity in an animal model. Transfection of EMMPRIN cDNA or EMMPRIN/GFP cDNA into human MDA-MB-436 breast cancer cells resulted in marked enhancement of tumor growth in nude mice after orthotopic injection of tumor cells as compared to injection of vector or GFP alone-transfected tumor cells. High levels of gelatinase A and gelatinase B mRNA expression were demonstrated by in situ hybridization in EMMPRIN-transfected tumors as compared to vector or GFP-transfected tumors. Regardless of the size of the tumors, enhanced gelatinase B and gelatinase A levels were identified in zymograms from extracts of EMMPRIN/GFP-transfected tumors as compared to GFP tumors. The 105 kDa gelatinolytic band represents mouse gelatinase B,[25] but the 92 kDa band could be either activated mouse gelatinase B or latent human gelatinase B.

It is noteworthy that human MDA-MB-436 cells propagated in vitro readily secreted progelatinase A, but secreted minimal gelatinase B, whereas extracts of tumors removed from nude mice injected with these tumor cells contained higher levels of gelatinase B than gelatinase A. Treatment of these breast cancer cells with thrombin and PMA in vitro resulted in increased secretion and activation of progelatinase A and progelatinase B, respectively; which is consistent with the stimulatory effects of these agents described with other types of cells.[22,26] These observations are consistent with the concept that both mouse host cells and transplanted human cancer cells are responsible for the production of gelatinase A and gelatinase B in nude mouse tumors. An association between expression of EMMPRIN and gelatinase B in benign and malignant pigment cell skin lesions in humans has been reported,[27] but a direct stimulatory effect of EMMPRIN on gelatinase B expression has not been previously described.

Another important observation in this study was that EMMPRIN-transfected MDA-MB-436 cancer cells secreted higher levels of gelatinase A in vitro than vector-transfected cells; this presumably represents autocrine stimulation. These cancer cells also displayed a more invasive

phenotype than control transfectants when examined in a modified Boyden chamber (manuscript submitted[1]).

Our in situ hybridization data of tumors transplanted into nude mice differs from de novo human breast cancer. Gelatinase A mRNA was identified both in EMMPRIN-transfected human cancer cells growing in nude mice and in surrounding host stromal cells (Figure 3). Previous studies in patients with breast cancer demonstrated the expression of gelatinase A and gelatinase B almost exclusively in peri-tumoral stromal and inflammatory cells, respectively. [6,8,28] However, a few reports have described gelatinase B expression in breast,[29] lung,[30] and liver carcinoma cells.[30,31] In comparing experimental cancer models to the human counterpart, it needs to be emphasized that cancer cell lines propagated in vitro e.g. MDA-MB-436, which are selected for their invasive properties, generally express high levels of gelatinases.[32] Furthermore, by comparison to in situ human breast cancers,[33] transplanted human tumors in nude mice demonstrate sparse inflammatory and fibrotic reactions; this represents an important distinction that is often overlooked. These differences between human and animal models of cancer need to be considered in predicting human responses to novel therapies developed in experimental animal models.

In one of three sets of experiments, metastasis following orthotopic injection of tumor cells into nude mice occurred more frequently with EMMPRIN-transfected cells than with vector-transfected cells, but the overall rate was low. As we previously reported,[34] EMMPRIN expression did not affect tumor cell proliferation in vitro. Based on the established role of EMMPRIN in enhancing MMP synthesis by stromal cells, it would appear that increased degradation of extracellular matrix permits more rapid tumor growth in vivo. The higher rate of tumor growth with EMMPRIN-transfected cancer cells and the associated matrix degradation may also occur by favored neoplastic cell survival in a tissue stroma environment initially not permissive for tumor growth. Enhanced extracellular matrix degradation may also release growth

[1] Caudroy S., Polette M.,Nawrocki-Raby B., Toole B., Zucker S., Birembaut P. EMMPRIN-induced invasiveness of a human mammary tumor cell line is associated with enhancement of matrix metalloproteinase-2 expression and activity.

factor-like fragments of matrix components, resulting in an indirect effect on cell proliferation.[35] A role for host-derived MMPs in tumor progression and angiogenesis has been supported by studies in gelatinase A-deficient (knockout) mice.[36] In contrast to these findings with EMMPRIN, stromelysin-3 (an MMP with minimal proteolytic activity on ECM proteins) expression in cancer cells promoted tumor take, but not tumor growth in nude mice.[37] These studies with EMMPRIN reinforce the notion that cancer dissemination is a multistep process and that extracellular matrix degradation contributes to the process but is insufficient in itself to account for tumor metastasis.[38] Continued exploration of genes responsible for the metastatic process is warranted.[39]

ACKNOWLEDGMENTS

This article is dedicated to the memory of our friend and colleague, Chitra Biswas, whose career was dedicated to the discovery and exploration of EMMPRIN. Dr. Biswas died in August 1993, but her inspiration continues to guide us in our studies of EMMPRIN.

We thank Dr. Serge Lyubsky for his contribution to the histopathologic studies.

FIGURE LEGENDS

Figure 1A. Schematic illustration of the EMMPRIN/GFP plasmid. A 1.6 kb cDNA representing the entire EMMPRIN sequence was placed at an EcoR 1 site under the control of the CMV promoter in pcDNA 3. GFP cDNA was inserted along with an upstream CMV promoter into the EMMPRIN expression vector between Not I and Xho I sites. A polyadenylation (PA) signal was placed downstream.

Figure 1B. Northern blot analysis of EMMPRIN. ~20 μg of total cellular RNA from plasmid alone-transfected, GFP-transfected, and EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells (from experiment 3) was size fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 1.7 kb of ^{32}P-radiolabeled EMMPRIN cDNA as a probe. Blots were analyzed by autoradiography. A single 1.7 kb mRNA transcript corresponding to the known EMMPRIN band was detected at ~20 X greater intensity in EMMPRIN/GFP transfected cells as compared to plasmid alone or GFP transfected cells.

Figure 2A. MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA resulted in enhanced rate of tumor growth after tumor cell implantation into the mammary fat pad of nude mice as compared to GFP-transfected cells. The tumorigenicity of transfected cells was assessed by weekly measurement of tumor size. The data represents the mean ± standard error observed in 10 animals in each group injected with 1 X 10^6 transfected cancer cells. The numbers associated with each symbol refer to the number of mice alive at each time point (i.e. at week 8, 3 mice in the EMMPRIN/GFP group had large tumors and were sacrificed, hence the number 7 is listed).

Figure 2B. GFP-transfected tumors are readily visible under fluorescent light. EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells were injected into the mammary tissue of a female NCr nu/nu mouse. Eight weeks later, the mouse was sacrificed and extensive green colored metastatic tumors in the peritoneum, liver, spleen, and mediastinum were visible under

fluorescent light (left hand photo). The photo on the right demonstrates the same tumors visualized by bright light (arrowheads identify tumors).

Figure 2C. Comparison of gelatinases secreted by MDA-MB-436 cells cultivated in serum-free media and extracts of nude mouse tumors. Spent serum-free conditioned media from primary cells cultivated for 18 hours with vehicle (media), PMA, and thrombin (left panel) and tumor cell extracts (right panel) were assessed by gelatin substrate zymography. Protein concentration (15 ug/well) of tissue samples were equalized within each group. Conditioned media and tumor extracts from the EMMPRIN/GFP group displayed more gelatinolytic activity than the GFP-alone group of mice. The displayed extract from the GFP-alone tumor is from the largest tumor (1.4 cm^3) in this group of mice. The intensity of tumor gelatinolytic activity demonstrated in each group of mice did not correlate with tumor size (data not shown). Molecular weights were calculated using protein standards. The conditioned media of HT-1080 cells was utilized to confirm the molecular weight of human gelatinase A and gelatinase B (data not shown).

Figure 3. In situ hybridization of primary tumors from mice injected with EMMPRIN/GFP and GFP-transfected MDA-MB-436 breast cancer cells. Serial sections from tumor tissue (panels 1-4, 9-12) and surrounding non malignant tissue (panels 5-8 and 13-16) were examined. Panels 1 and 9 represent hematoxylin and eosin staining of cancer tissues from EMMPRIN/GFP and GFP tumors, respectively; panels 5 and 13 represent H & E staining of non malignant mammary tissues (tumor cells not identified) adjacent to the primary EMMPRIN/GFP and GFP tumors, respectively. Cells in the primary tumor mass from mice injected with EMMPRIN/GFP transfected cells revealed widely distributed, specific staining with EMMPRIN, gelatinase A (GLA), and gelatinase B (GLB) antisense riboprobes (panels 2-4, respectively). Minimal cell staining for EMMPRIN, gelatinase A, and gelatinase B was seen in cancer cells from GFP-transfected MDA-MB-436 cells (panels 10-12). Non malignant tissues adjacent to the primary

REFERENCES

1. Biswas C, Zhang Y, DeCastro R, Guuo H, Nakamura T, Kataoka H, Nabeshima K: The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res. 1995, 55:434-439

2. Kataoka H, DeCastro R, Zucker S, Biswas C: The tumor cell-derived Collagenase Stimulating Factor, TCSP, increases expression of interstitial collagenase, stromelysin and 72 kDa gelatinase. Cancer Res. 1993, 53:3155-3158

3. Guo H, Zucker S, Gordon M, Toole BP, Biswas C: Stimulation of metalloproteinase production by recombinant EMMPRIN from transfected CHO cells. J. Biol. Chem. 1996, 272:24-27

4. Guo HL, Zucker S, Toole BP: EMMPRIN (CD147) an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. Cancer Res. 2000, 60:888-891

5. Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 1990, 348:699-704

6. Heppner KJ, Matrisian LM, Jensen RA, Rodgers WH: Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. Am. J. Pathol. 1996, 149:273-282

7. Polette M, Toole B, Tournier J-M, Zucker S, Birembaut P: TCSF expression and localization in human lung and breast cancer. J. Histochem. Cytochem. 1997, 45:703-709

8. Dalberg K, Eriksson E, Enberg U, Kjellman M, Backdahl M: Gelatinase A, membrane type 1 matrix metalloproteinase, and extracellular matrix metalloproteinase inducer mRNA expression: correlation with invasive growth of breast cancer. World J. Surgery 2000, 24:334-340

9. Nabeshima K, Lane WS, Biswas C: Partial sequencing and characterization of the rumor cell-derived collagenase stimulatory factor. Arch. Biochem. Biophys. 1991, 285:90-96

10. Miyauchi T, Masuzawa Y, Muramatsu T: The basigin group of the immunoglobulin superfamily: Complete conservation of a segment in and around transmembrane domains of human and mouse basigin and chicken HT7 antigen. J. Biochem. 1991, 110:770-774

11. Kasinrerk W, Fiebiger E, Steffanova I, Baumruker T, Knapp W, Stockinger H: Human leukocyte activation antigen M6, a member of the Ig superfamily, is the species homologue of rat OX-47, mouse basigin, and chicken HT7 molecule. J. Immunol. 1992, 149:847-854

12. Igakara T, Kadomatsu K, Kaname T, Muramatsu T, Fan Q-W, Yuasa S, Takahashi M, Senda T, Taguchi O, Yamamura KI, Arumura K, Muramatsu T: A null mutation in basigin, an immunoglobulin superfamily member, indicates important role in peri-implantation andspermatogenesis. Devel. Biol. 1998, 194:152-163

13. Thompson EW, Paik S, Brunner N, Sommers CL, Zugmaier G, Clarke R, Shima TB, Torri J, Konahue S, Lipman ME, Martin GM, Dickson. RB: Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimetin in human breast cancer cell lines. J. Cell Physiol. 1992, 150:534-544

14. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker of gene expression. Science 1994, 263:802-805

15. Ellis SM, Nabishima K, Biswas C: Monoclonal antibody preparation and purification of a tumor cell collagenase-stimulating factor. Cancer Res. 1989, 49:3385-3391

16. Cao J, Sato J, Takino T, Seiki M: The C-terminal region of membrane type matrix metalloproteinase is a functional transmembrane domain required for progelatinase A activation. J. Biol. Chem. 1995, 270:801-805

17. Cao J, Rehemtulla A, Bahou W, Zucker S: Membrane type matrix metalloproteinase 1 (MT-MMP1) activates progelatinase A without furin cleavage of the N-terminal domain. J. Biol. Chem. 1996, 271:30174-30180

18. Birk PE, Grimm PC: Rapid nonradioactive in situ hybridization for interleukin-2 mRNA with riboprobes generated using the polymerase chain reaction. J. Immunol. Methods 1994, 167:83-89

19. Komminoth P: Detection of mRNA in tissue sections using DIG-labeled RNA and oligonucleotide probes. In S. Grunewald-Janho, J. Keesey, M. Leous, R. van Miltenburg, and Schroeder (Eds.). 1996, 126-135

20. Woessner JF: Quantification of matrix metalloproteinases in tissue samples. In Barrett AJ (ed): 'Proteolytic enzymes: Aspartic and Metallopeptidases, "San Diego: Academic Press. 1995, 510-528

21. Zucker S, Mirza H, Conner C, Lorenz A, Drews M, Bahou WF, Jesty J: Vascular endothelial growth factor induces a tissue factor cascade in endothelial cells: Conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int. J. Cancer 1998, 75:780-786

22. Zucker S, Conner C, DiMassimo BI, Ende H, Drews M, Seiki M, Bahou WF: Thrombin induces the activation of progelatinase A in vascular endothelial cells: Physiologic regulation of angiogenesis. J. Biol. Chem. 1995, 270:23730-23738

23. Tsuruga E, Sakakura Y, Yajima T, Shide N: Appearance and distribution of dendritic cells and macrophages in dental pulp during early postnatal morphogenesis of mouse mandibular first molars. Histochem. Cell Biol. 1999, 112:193-204

24. Bourguignon LYW, Gunja-Smith Z, Iida N, Zhu HB, Young LJT, Muller WJ, Cardiff RD: CD44v3, 8-10 is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. J. Cell Physiol. 1998, 176:206-215

25. Tanaka H, Hojo K, Yoshida H, Yoshioka T, Sugita K: Molecular cloning and expression of the mouse 105-kDa gelatinase cDNA. Biochem. Biophys. Res. Commun. 1993, 190:732-740

26. Foda HD, George S, Conner C, Drews M, Tompkins DC, zucker S: Activation of human umbilical vein endothelial cell progelatinase A by phorbol myristate acetate (PMA): A protein kinase C-dependent mechanism involving a membrane-type matrix metalloproteinase. Lab. Invest. 1996, 74:538-545

27. van den Oorg JJ, Paemen L, Opdenakker G, De Wolf-Peeters C: Expression of gelatinase B and the extracellular matrix metalloproteinase inducer EMMPRIN in benign and malignant pigment cell lesions of the skin. Amer. J. Pathol. 1997, 151:665-670

28. Polette M, Birembaut P: Matrix metalloproteinases in breast cancer. The Breast Journal 1996, 2:209-220

29. Soini Y, Hurskainen T, Hoyhtya M, Oikarinen A, Autio-Harainen H: 72 kD and 92 kD type IV collageanse, type IV collagen and laminin mRNAs in breast cancer: A study by in situ hybridization. J. Histochem. Cytochem. 1994, 42:945-951

30. Canete-Soler R, Litzky L, Lubenski I, Muschel RJ: Localization of the 92 kd gelatinase mRNA in squamous cell and adenocarcinomas of the lung using in situ hybridization. Am. J. Pathol. 1994, 144:518-527

31. Ashida K, Nakatsukasa H, Higashi T: Cellular distribution of 92-kDa typeIV collagenase/gelatinase B in human hepatocellular carcinoma. Am. J. Pathol. 1996, 149:1803-1818

32. Giambernardi TA, Grant GM, Taylor GP, Hay RJ, Maher VM, McCormick JJ, Klebe RJ: Overview of matrix metalloproteinase expression in cultured human cells. Matrix Biol. 1998, 16:483-496

33. Noel A, Munaut C, Nusgens B, Lapiere CM, Foidart JM: Different mechanisms of extracellular matrix remodeling by fibroblasts in response to human mammary neoplastic cells. Invasion Metastasis 1993, 13:72-81

34. Zucker S, Biswas C: Tumor collagenase stimulating factor (TCSF): A paracrine stimulating factor of fibroblast production of matrix metalloproteinases in cancer. Bulletin de l'Institut Pasteur 1994, 92:282-290

35. Chambers AF, Matrisian L: Changing view of the role of matrix metalloproteinases in metastasis. J. Natl. Cancer Inst. 1997, 89:1260-1270

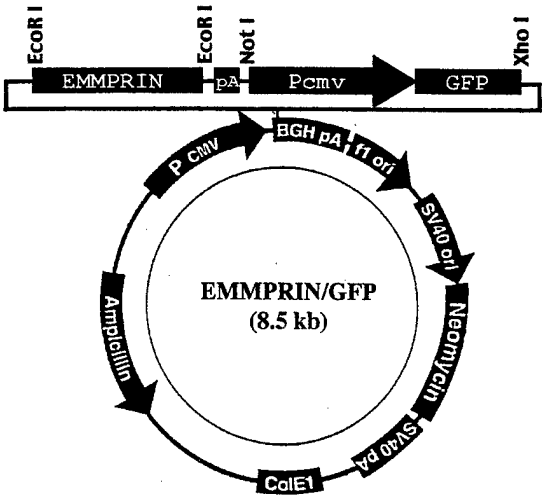
36. Itoh T, Tanioka M, Yoshida H, Nishimoto H, Itohara S: Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res. 1998, 58:1048-1051

37. Noel AC, Lefebre O, Maquoi E, Van Hoorde L, Chenard MP, Mareel M, Foidard J-M, Basset P, Rio M: Stromelysin-3 expression promotes tumor take in nude mice. J. Clin. Invest. 1996, 97:1924-1930

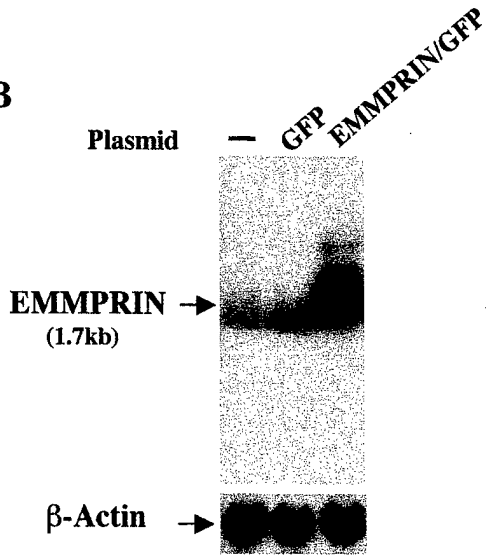
38. Witty JP, McDonnell S, Newell KJ, Cannon P, Navre M, Tressler RJ, Matrisian LM: Modulation of matrilysian levels in colon carcinoma cell lines affects tumorigenicity in vitro. Cancer Res. 1994, 54:4805-4812

39. Clark EA, Golub TR, Lander ES, Hynes RO: Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000, 406:532-535

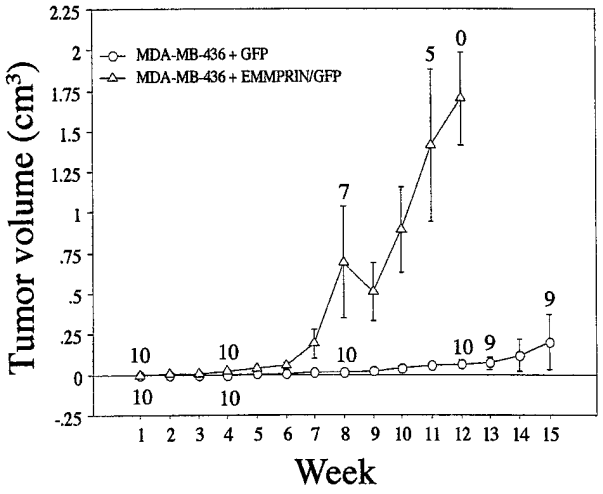
A


EcoR I
EcoR I
Not I
Xho I
EMMPRIN
pA
Pcmv
GFP
P CMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
ColE1
Ampicillin
EMMPRIN/GFP
(8.5 kb)

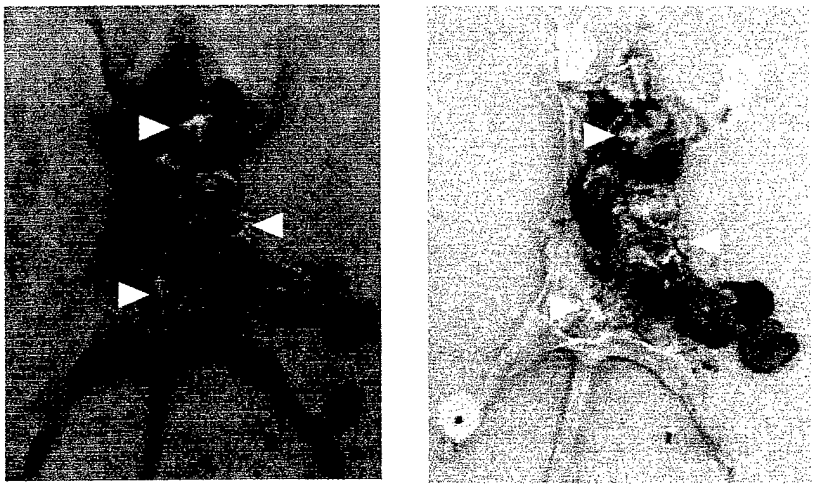
B


Plasmid
—
GFP
EMMPRIN/GFP
EMMPRIN
(1.7kb)
β-Actin

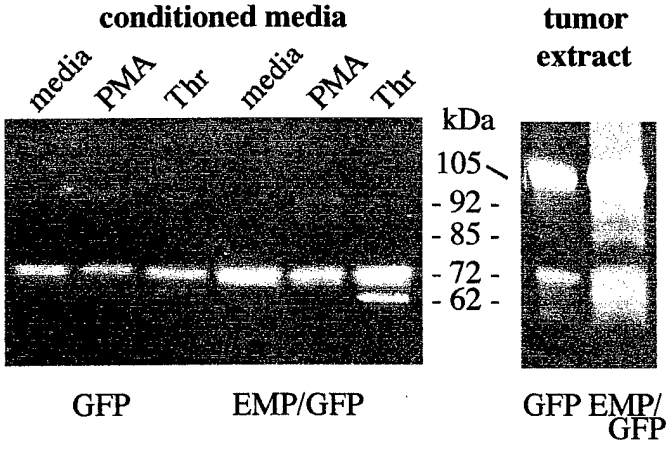
A

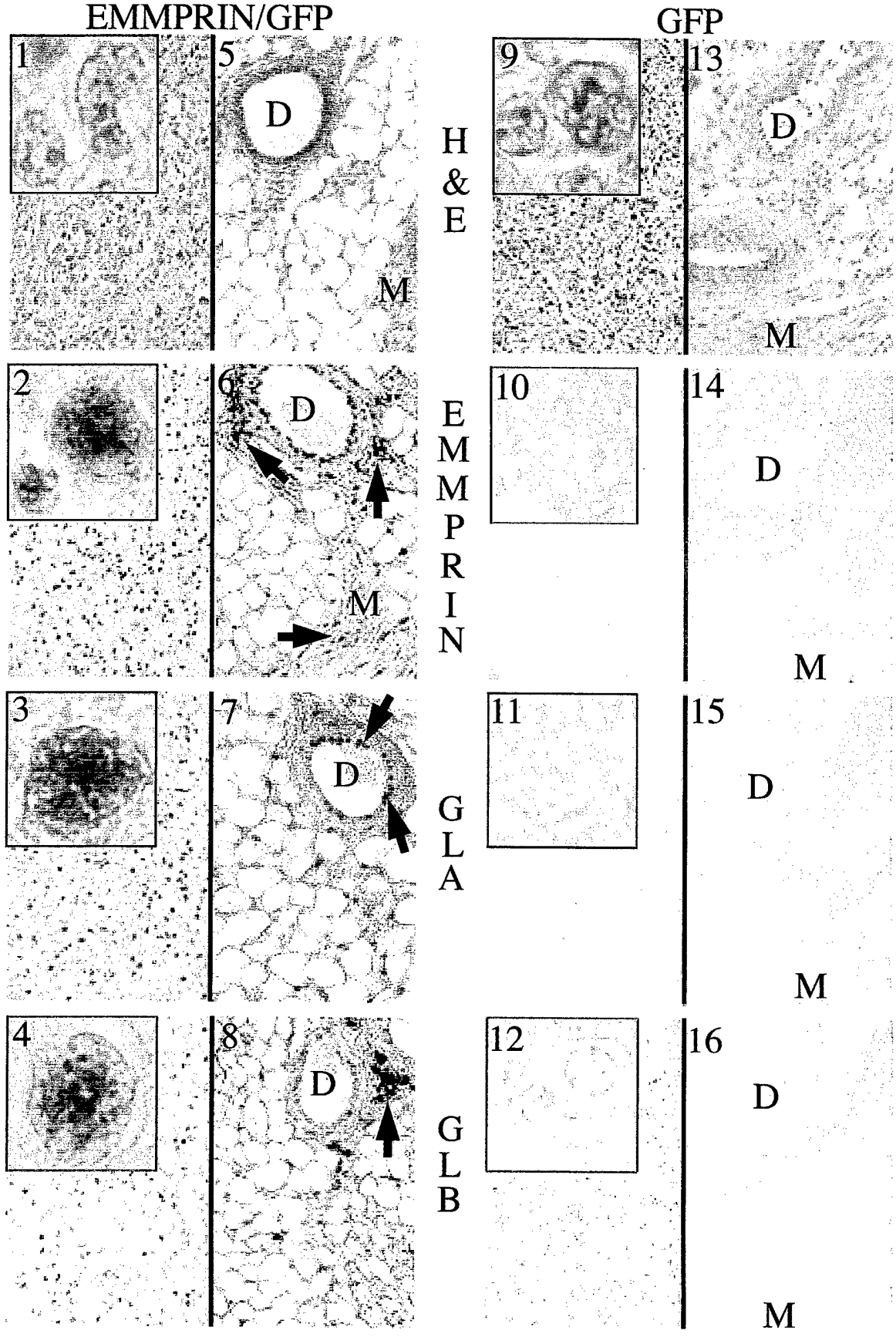

MDA-MB-436 + GFP
MDA-MB-436 + EMMPRIN/GFP
Tumor volume (cm3)
Week
2.25
2
1.75
1.5
1.25
1
.75
.5
.25
0
-.25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
10
10
10
10
10
10
10
7
5
0
9

B



C


conditioned media
tumor extract
media
PMA
Thr
media
PMA
Thr
kDa
105
92
85
72
62
GFP
EMP/GFP
GFP
EMP/GFP


EMMPRIN/GFP
GFP
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
D
M
H & E
EMMPRIN
GLA
GLB

[CANCER RESEARCH 60, 888–891, February 15, 2000]

Advances in Brief

APPENDIX 3

EMMPRIN (CD147), an Inducer of Matrix Metalloproteinase Synthesis, Also Binds Interstitial Collagenase to the Tumor Cell Surface[1]

Huiming Guo, Rongsong Li, Stanley Zucker, and Bryan P. Toole[2]

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111 [H. G., R. L., B. P. T.], and Departments of Research and Medicine, Veteran Affairs Medical Center, Northport, New York 11768 [S. Z.]

Abstract

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells. In this study, we have found that EMMPRIN not only stimulates the production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface. Complex formation was demonstrated by phage display, affinity chromatography, and immunocytochemistry. Presentation of MMP-1 complexed to EMMPRIN at the tumor cell surface may be important in modifying the tumor cell pericellular matrix to promote invasion.

Introduction

MMPs[3] have been implicated in several aspects of tumor progression, including invasion through basement membranes and interstitial matrices, angiogenesis, and tumor cell growth (1–3). Strong support for the involvement of MMPs at some step in tumor progression comes from experiments in which tissue inhibitors of MMPs or synthetic inhibitors of metalloproteinases have been shown to reduce tumor growth and metastasis (4, 5). Over the past several years, it has become increasingly apparent that tumor cells create a pericellular environment in which MMPs and other proteases become concentrated, thus enhancing the ability of tumor cells to invade extracellular matrices (6–8). Previous studies from this laboratory have demonstrated that EMMPRIN, a member of the immunoglobulin superfamily that is enriched on the surface of most tumor cells, stimulates stromal cells to produce elevated levels of several MMPs, including MMP-1 (9–11). We have now found that tumor cell EMMPRIN not only stimulates MMP-1 production by fibroblasts but also binds MMP-1 to the surface of tumor cells, thus adding to the complement of proteases on the tumor cell surface that may promote invasion.

Materials and Methods

Phage Display Library. mRNA was prepared from human fibroblasts with the Oligotex mRNA kit (Qiagen, Valencia, CA) and used for cDNA synthesis with the Directional RH primer cDNA synthesis kit (Novagen, Madison, WI). After second-strand synthesis, the cDNA ends were flushed with T4 DNA polymerase and ligated to *Eco*RI/*Hin*dIII directional linkers. The cDNA was then digested with *Eco*RI and *Hin*dIII and ligated to T7Select1-1b vector arms (Novagen). The ligated DNA was packaged into bacteriophage T7 using the T7Select1-1 Packaging Extract (Novagen). The host strain of bacteria, BLT 5403 (Novagen), was then grown to $A_{600\ nm}$ = 0.8–1.0 and mixed with the packaged cDNA (at a ratio of 10^6 phage/10 ml cells) in LB media containing 50 μg/ml carbenicillin (Novagen). Molten top agarose at 45°C–50°C was added to the phage/host mixture (10:1) and immediately poured onto a 150-mm plate containing LB/carbenicillin medium. The plate was incubated at room temperature overnight until the plaques were nearly confluent. The phage was then eluted by covering the plate with phage extraction buffer [100 mM NaCl, 20 mM Tris, and 6 mM $MgSO_4$ (pH 8.0)] at 4°C overnight. The phage lysate was clarified with chloroform and subjected to screening by biopanning.

Screening of Phage Display Library. Twenty four-well cell culture plates were prepared for biopanning as suggested by the manufacturer (Novagen). The wells were coated with immunopurified EMMPRIN protein (Ref. 12; 1 μg/ml in Tris-buffered saline) at 4°C overnight and washed with Tris-buffered saline five times. Unreacted sites were blocked with 5% blocking reagent overnight at 4°C and washed. In the first round of screening, the phage lysate was applied to the EMMPRIN-coated plate (0.5 ml lysate/well) for 30 min at room temperature. The plate was then washed five times with Tris-buffered saline. The bound phages were eluted by adding 0.5 ml of elution buffer (1% SDS) at room temperature for 20 min. The eluted phages were then added to a culture of the host cells (BLT 5403) in LB media and incubated at 37°C with shaking for 3 h, at which time lysis was observed. The lysed culture was centrifuged, and the supernatant was collected for the next round of biopanning. A total of five rounds of screening was carried out. DNA from the phages isolated during the final round of screening was purified and sequenced using the T7 SelectUp primer (GGAGCTGTCGTATTCCAGTC) and the T7 SelectDown primer (AACCCTCAAGACCCGTTTA; Novagen).

Immunoaffinity and Ligand Affinity Chromatography. EMMPRIN was isolated from extracts of membranes from LX-1 human lung carcinoma cells by immunoaffinity chromatography using E11F4 monoclonal antibody against EMMPRIN immobilized on Sepharose beads, as described previously (12).

For manufacture of the ligand affinity medium, EMMPRIN protein (0.5 mg) was first dissolved in coupling buffer [0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3) containing 0.5% NP40]. The coupling solution was then mixed with CNBr-activated Sepharose 4B gel (Pierce; 0.25 g of dried powder swelled and washed in 1 mM HCl for 30 min) at 4°C. After overnight incubation, the gel was washed three times with 5 ml of coupling buffer, followed by incubation in 0.1 M Tris-HCl (pH 8) for 2 h to block any remaining active groups. Then the gel was washed using three cycles of 0.1 M acetate buffer, 0.5 M NaCl (pH 4), and 0.1 M Tris and 0.5 M NaCl (pH 8). After washing, the gel was resuspended in 5 ml of 10 mM Tris buffer (pH 8.3).

Extracts of human fibroblasts [10^8 cells in 5 ml of 10 mM Tris, 0.15 M NaCl, and 0.5% NP40 (pH 8.3)] were added to the EMMPRIN-coupled gel and incubated at 4°C overnight with rotation. The gel was then washed with 10 mM Tris and 0.15 M NaCl containing 30 mM octyl glucoside until the $A_{280\ nm}$ was less than 0.05. Binding proteins were eluted with 0.1 M glycine buffer (pH 2.5) containing 30 mM octyl glucoside. The eluate was neutralized to pH 7 by the addition of 1 M Tris (pH 9.5) and concentrated for further analysis.

ELISA of MMP-1. MMP-1 protein was measured in the eluates from EMMPRIN-Sepharose and in immunopurified EMMPRIN preparations using a commercial ELISA system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Briefly, 5 or 10 μl of eluate were added to microtiter plates coated with antibody to MMP-1 and incubated for 2 h at 25°C. The plates were washed with phosphate buffer and incubated with anti-MMP-1 antiserum for 2 h. After washing, the plates were incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for color development and measurement at $A_{450\ nm}$ in a microplate spectrophotometer. The concentration of MMP-1 in the eluate was estimated from a standard curve.

Received 11/18/99; accepted 1/3/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by United States Army Grants DAMD17-95-1-5017 and DAMD17-99-9413.

[2] To whom correspondence should be addressed. Fax: (617) 636-0380; E-mail: btoole@infonet.tufts.edu.

[3] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; MMP-1, interstitial collagenase; MMP-2, gelatinase A; MT-MMP, membrane-type MMP; LB, Luria-Bertani.

SDS-PAGE, Silver Staining, and Western Blotting. Proteins were dissolved in SDS sample buffer containing 0.1 M DTT and heated at 95°C for 5 min. The samples were then subjected to electrophoresis on 10% SDS polyacrylamide gels. The gels were either stained using the Sterling silver staining system (National Diagnostics, Atlanta, CA) or electroblotted onto nitrocellulose membranes and incubated with antibody against EMMPRIN (E11F4; Ref. 12) or against MMP-1 (Calbiochem, La Jolla, CA) for 1 h at room temperature. The immunoreactive protein bands were detected with horseradish peroxidase-conjugated antimouse IgG and chemiluminescence reagent (New England Nuclear Life Science, Boston, MA).

Immunocytochemistry. LX-1 human lung carcinoma cells were seeded into chamber culture slides and cultured for 48 h at 37°C in 5% CO_2 air. The cells were then washed with PBS, fixed in 1% paraformaldehyde in PBS for 45 min at room temperature, quenched with 0.1 M Tris (pH 7.4), and blocked with 1% BSA, 1% goat serum, and 2% nonfat milk in PBS at room temperature for 1 h. The LX-1 cells were then incubated with monoclonal antibody against MMP-1 (Calbiochem) for 1 h at room temperature, followed by Cy3-conjugated Texas red goat antimouse IgG. The cells were washed with PBS, mounted with coverslips, and then observed and photographed using a Zeiss Axioskop-20 microscope.

Results

Phage Display Reveals MMP-1 as an EMMPRIN-binding Protein. We used the T7Select Phage Display System (Novagen) to identify EMMPRIN-binding protein(s) encoded by a cDNA library prepared from human fibroblasts, as described in "Materials and Methods." In this method, each phage becomes coated with a fusion protein comprised of the phage coat protein and a protein generated from the cDNA library used. Phages coated with putative EMMPRIN-binding protein were selected by repeated panning over 24-well plates coated with EMMPRIN. Five rounds of biopanning were carried out, and the final lysate was used for plaque assay, PCR amplification, and sequencing.

Eight clones were obtained from the procedure described above. All eight of the inserts were of identical size, *i.e.*, 0.8 kb, and were found to have identical sequences corresponding exactly to a portion of the human MMP-1 sequence (Fig. 1).

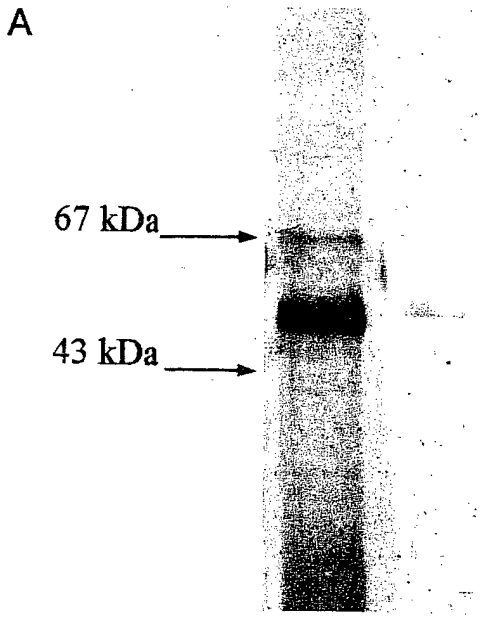
MMP-1 Binds to EMMPRIN-Sepharose. To confirm the binding of EMMPRIN to fibroblast-produced MMP-1, we performed ligand chromatography over Sepharose conjugated with immunopurified EMMPRIN. Fibroblast extracts were mixed with the EMMPRIN-Sepharose, which was then washed and eluted as described in "Materials and Methods." The eluates were subjected to SDS-PAGE, followed by silver staining. On silver staining, a prominent protein band at ~M_r 55,000 was observed, as well as a weaker band at ~M_r 67,000 (Fig. 2*A*); in some cases a ~M_r 45,000 band could also be seen.

Western blots were also performed on the eluates from EMMPRIN-Sepharose using antibody against human MMP-1. The protein band at ~M_r 55,000 (the approximate size of pro-MMP-1, which is M_r 52,000) reacted with anti-MMP-1 antibody (Fig. 2*B*), confirming our results from the phage display. ELISA measurements also revealed MMP-1 in the eluates from EMMPRIN-Sepharose (data not shown). The identities of the ~M_r 67,000 and ~M_r 45,000 proteins are not yet known.

EMMPRIN Forms a Complex with MMP-1 on the Surface of Tumor Cells. Some tumor cells themselves produce small amounts of MMP-1. Thus, we also determined whether, in addition to binding isolated EMMPRIN protein, MMP-1 forms a complex with EMMPRIN present on the surface of LX-1 human lung carcinoma cells. We immunopurified EMMPRIN from extracts of LX-1 cell membranes using monoclonal antibody E11F4 covalently bound to Sepharose beads and tested whether MMP-1 was present in the eluted EMMPRIN preparation. Fig. 3 shows a Western blot of such an

```
                1                                                        55
5' sequence:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                941                                                     995

                56                                                      110
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                996                                                     1050

                111                                                     165
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                1051                                                    1105


                1                                                        55
3' sequence:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                343                                                     397

                56                                                      110
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                398                                                     452

                111                 131
                TGTGGACCATGCCATTGAGAA
                |||||||||||||||||||||
                TGTGGACCATGCCATTGAGAA
                453                 473
```

Fig. 1. Comparison of partial sequences of cDNA isolated by phage display with that of human MMP-1 cDNA. The 5′ and 3′ sequences of one of the partial cDNAs obtained are given. Eight cDNA clones were isolated after biopanning of phages on EMMPRIN; all eight clones had identical sequences.



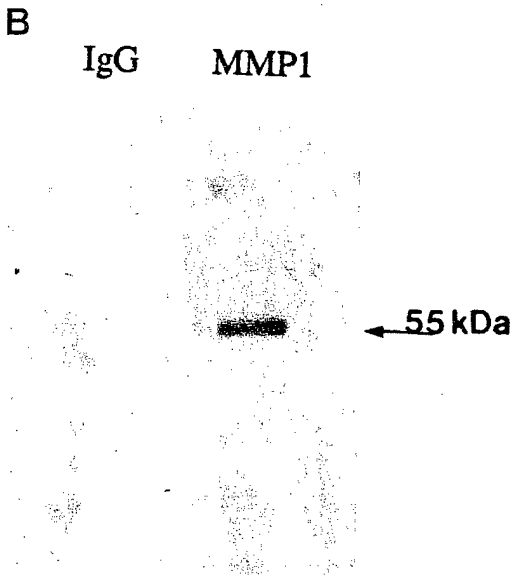


Fig. 2. EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. *A*, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at ~M_r 55,000 and ~M_r 67,000) were detected. *Arrows* indicate positions of the M_r 43,000 and M_r 67,000 markers. *B*, parallel gels to those in *A* were transblotted and reacted with antibody to MMP-1 or secondary antibody only (*IgG*); the ~M_r 55,000 band reacted with anti-MMP-1.

EMMPRIN preparation with antibody against MMP-1. A strong band at ~M_r 55,000, corresponding approximately in size to pro-MMP-1, reacted with the antibody, indicating the presence of MMP-1 in the EMMPRIN preparation. A weaker band at ~M_r 45,000, which is not seen consistently, is most likely activated MMP-1 (M_r 42,000).

Quantitation of the MMP-1 content by ELISA gave 2.1 μg of MMP-1 per 5 μg of total protein in the EMMPRIN preparation. Because EMMPRIN and pro-MMP-1 have molecular weights of ~58,000 and 52,000, respectively, this result suggests that EMMPRIN and MMP-1 are complexed in an equimolar ratio.

The presence of MMP-1 at the surface of LX-1 human lung carcinoma cells was confirmed by immunocytochemistry using antibody against MMP-1 (Fig. 4).

Discussion

Many recent studies have highlighted the importance of the pericellular milieu surrounding tumor cells in their proliferative and invasive behavior (6–8). This milieu is modified by a number of proteases, especially MMPs and tissue serine proteases, many of which are produced by tumor-associated stromal cells rather than tumor cells themselves (13, 14) and subsequently become concentrated at the tumor cell surface via interaction with specific binding

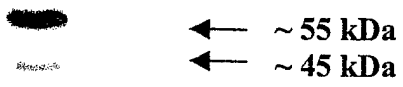


Fig. 3. Western blot of immunopurified EMMPRIN with antibody to MMP-1.

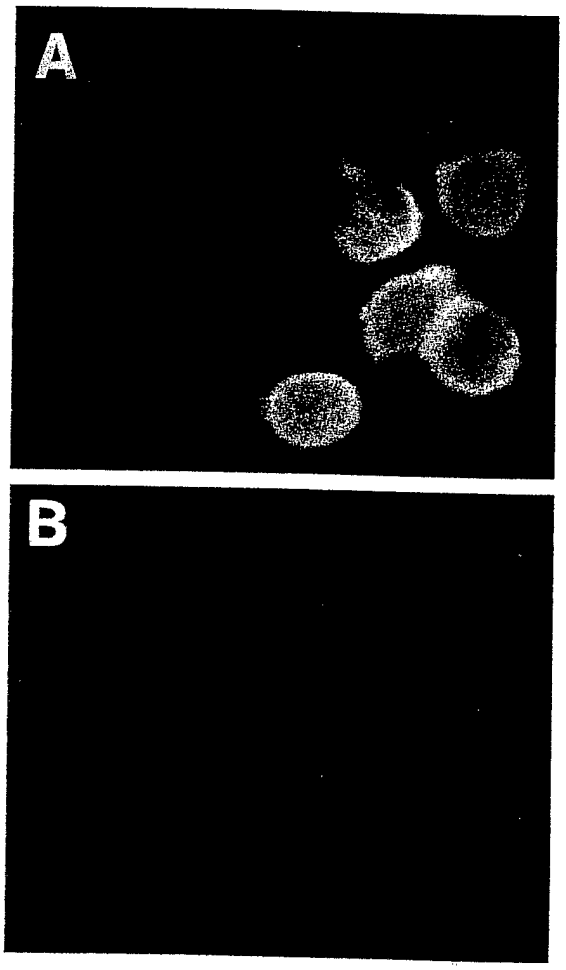


Fig. 4. Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. *A*, cells stained with antibody against MMP-1. *B*, cells stained with secondary antibody only.

sites. For example, MMP-2 binds to the tumor cell surface via a tissue inhibitor of MMPs-2-MT-MMP complex (15, 16). MMP-2 is activated by the MT-MMP, and the complex is targeted to invasive domains of the tumor cell membrane (sometimes termed "invadopodia") via specific docking of MT-MMP at these sites (17). Although MT-MMPs activate soluble MMP-2 as well as plasma membrane-retained MMP-2, membrane-bound enzyme is required for tumor cell invasion (17). A similar mechanism of activation and retention at the cell surface has been described for collagenase 3 (18). Other cell surface binding sites have been described for gelatinase B, *i.e.*, CD44 (19) and the α_2(IV) chain of collagen (20), and for MMP-2, *i.e.*, $\alpha_V\beta_3$ integrin (21). These sites also appear to be important in tumor cell invasion.

Evidence for association of MMP-1 with the surface of a human pancreatic carcinoma cell line has been published previously (22), but the mechanism whereby MMP-1 binds to these cells has not been described. In the present study, we show that MMP-1 binds to EMMPRIN, a tumor cell surface glycoprotein previously shown to induce synthesis of MMP-1 and other MMPs by fibroblasts (9–11) and endothelial cells.[4] We have also shown that an EMMPRIN-MMP-1 complex can be isolated from LX-1 human lung carcinoma cell membranes and that MMP-1 is present on the LX-1 cell surface. A preliminary report has been published suggesting that EMMPRIN becomes localized to invadopodia in human breast carcinoma cells (23). Tumor cell surface EMMPRIN may then be responsible for targeting MMP-1 to invadopodia, thus adding MMP-1 to the impressive list of proteases associated with these invasive structures (6, 17). Although other proteases have been shown to be important in tumor growth and invasion under a variety of conditions, it is likely that MMP-1 is crucial for penetration of fibrous tissues because of its ability to degrade fibrillar collagen as shown, for example, in endothelial cell invasion (24) and tumor cell invasion (25) of collagen gels. Thus localization of MMP-1 on the tumor cell surface via interaction with EMMPRIN would facilitate these invasive processes.

References

1. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, S. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, *64:* 327–336, 1991.
2. Chambers, A. F., and Matrisian, L. M. Changing views of the role of matrix metalloproteinases in metastasis. J. Natl. Cancer Inst., *89:* 1260–1270, 1997.
3. Shapiro, S. D. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr. Opin. Cell Biol., *10:* 602–608, 1998.
4. Khokha, R. Suppression of the tumorigenic and metastatic abilities of murine B16–F10 melanoma cells *in vivo* by the overexpression of the tissue inhibitor of matrix metalloproteinases-1. J. Natl. Cancer Inst., *86:* 299–304, 1994.
5. Eccles, S. A., Box, G. M., Court, W. J., Bone, E. A., Thomas, W., and Brown, P. D. Control of lymphatic and hematogenous metastasis of a rat mammary carcinoma by the matrix metalloproteinase inhibitor batimastat (BB-94). Cancer Res., *56:* 2815–2822, 1996.
6. Chen, W. T. Proteases associated with invadopodia, and their role in degradation of extracellular matrix. Enzyme Protein, *49:* 59–71, 1996.
7. Basbaum, C. B., and Werb, Z. Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. Curr. Opin. Cell Biol., *8:* 731–738, 1996.
8. Werb, Z. ECM and cell surface proteolysis: regulating cell ecology. Cell, *91:* 439–442, 1997.
9. Biswas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nabeshima, K. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res., *55:* 434–439, 1995.
10. Guo, H., Zucker, S., Gordon, M. K., Toole, B. P., and Biswas, C. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J. Biol. Chem., *272:* 24–27, 1997.
11. Lim, M., Martinez, T., Jablons, D., Cameron, R., Guo, H., Toole, B., Li, J., and Basbaum, C. Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. FEBS Lett., *441:* 88–92, 1998.
12. Ellis, S. M., Nabeshima, K. N., and Biswas, C. Monoclonal antibody preparation and purification of a tumor cell collagenase stimulatory factor. Cancer Res., *49:* 3385–3391, 1989.
13. Heppner, K. J., Matrisian, L. M., Jensen, R. A., and Rodgers, W. H. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. Am. J. Pathol., *149:* 273–282, 1996.
14. Johnsen, M., Lund, L. R., Romer, J., Almholt, K., and Dano, K. Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. Curr. Opin. Cell Biol., *10:* 667–671, 1998.
15. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G., and Goldberg, G. I. Mechanism of cell surface activation of 72-kDa type IV collagenase. J. Biol. Chem., *270:* 5331–5338, 1995.
16. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerk, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J. P., and Cao, J. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type 1-matrix metalloproteinase 1 (MT1-MMP). J. Biol. Chem., *273:* 1216–1222, 1998.
17. Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y., and Chen, W. T. Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloprotease docking to invadopodia is required for cell invasion. Proc. Natl. Acad. Sci. USA, *94:* 7959–7964, 1997.
18. Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase A (MMP-2) are able to generate active enzyme. J. Biol. Chem., *271:* 17124–17131, 1996.
19. Yu, Q., and Stamenkovic, I. Localization of matrix metalloproteinase-9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev., *13:* 35–48, 1999.
20. Olson, M. W., Toth, M., Gervasi, D. C., Sado, Y., Ninomiya, Y., and Fridman, R. High affinity binding of latent matrix metalloproteinase-9 to the α2(IV) chain of collagen IV. J. Biol. Chem., *273:* 10672–10681, 1998.
21. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$. Cell, *85:* 683–693, 1996.
22. Moll, U. M., Lane, B., Zucker, S., Suzuki, K., and Nagase, H. Localization of collagenase at the basal plasma membrane of a human pancreatic carcinoma cell line. Cancer Res., *50:* 6995–7002, 1990.
23. Chen, W. T., Goldstein, L. A., Pineiro-Sanchez, M., Howard, L., Ghersi, G., Nakahara, H., Salamone, M., Flessate, D., and Yeh, Y. Integral membrane proteases as potential diagnostic targets for breast malignancy. *In:* Proc. Department of Defense Breast Cancer Research Program Meeting, pp. 687–688. Fort Detrick, MD: Department of the Army, 1997.
24. Fisher, C., Gilberston-Beadling, S., Powers, E. A., Petzold, G., Poorman, R., and Mitchell, M. A. Interstitial collagenase is required for angiogenesis *in vitro*. Dev. Biol., *162:* 499–510, 1994.
25. Benbow, U., Schoenermark, M. P., Mitchell, T. I., Rutter, J. L., Shimokawa, K., Nagase, H., and Brinckerhoff, C. E. A novel host/tumor cell interaction activates matrix metalloproteinase 1 and mediates invasion through type I collagen. J. Biol. Chem., *274:* 25371–25378, 1999.

[4] Unpublished observations.